A transgenic mouse model engineered to investigate human brain-derived neurotrophic factor in vivo

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Editorial Manager(tm) for Transgenic Research
Manuscript Draft

Manuscript Number: TRAG342R1

Title: A transgenic mouse model engineered to investigate human brain-derived neurotrophic factor in vivo

Article Type: Original research papers

Section/Category:

Keywords: BDNF; neurotrophic factor; YAC; transgene; expression; reporter

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Manuscript Region of Origin:

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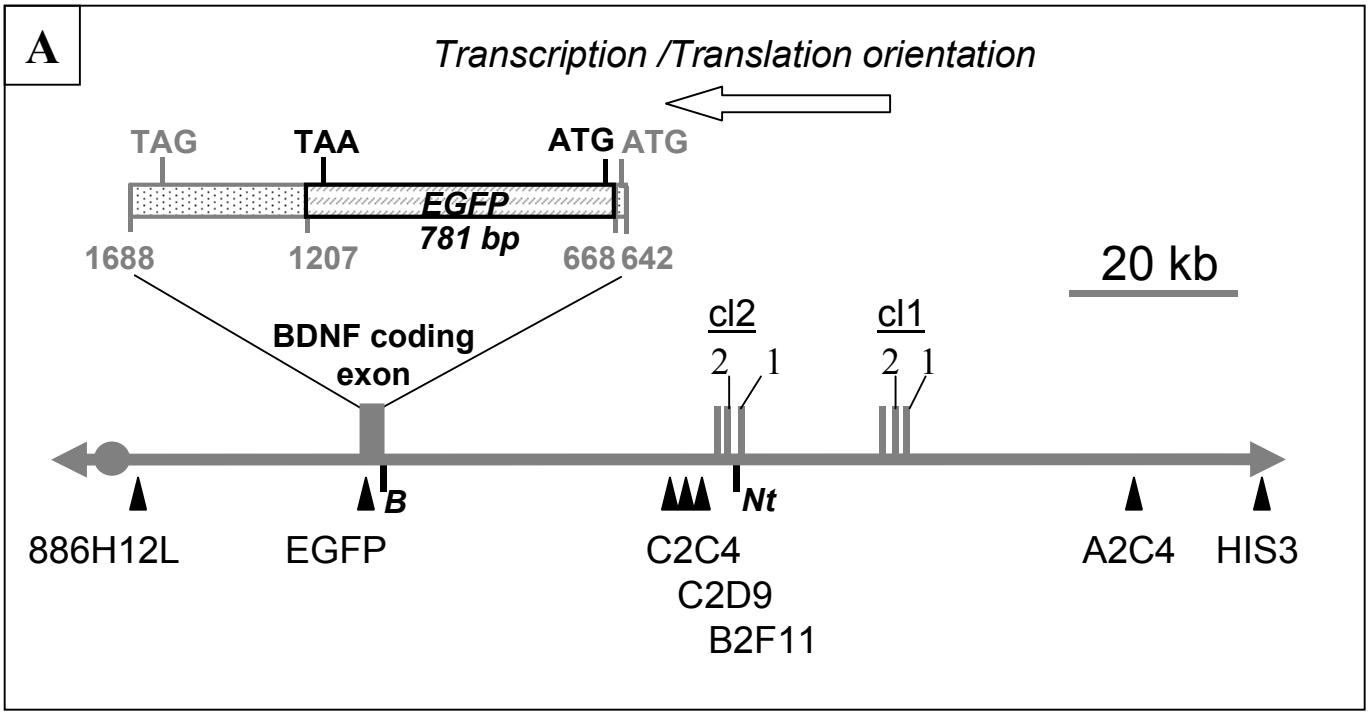
and localization of the fluorescent reporter gene product in the expected brain subregions are reported. This line constitutes an exploitable system for investigating human BDNF gene regulation in vivo.

line figure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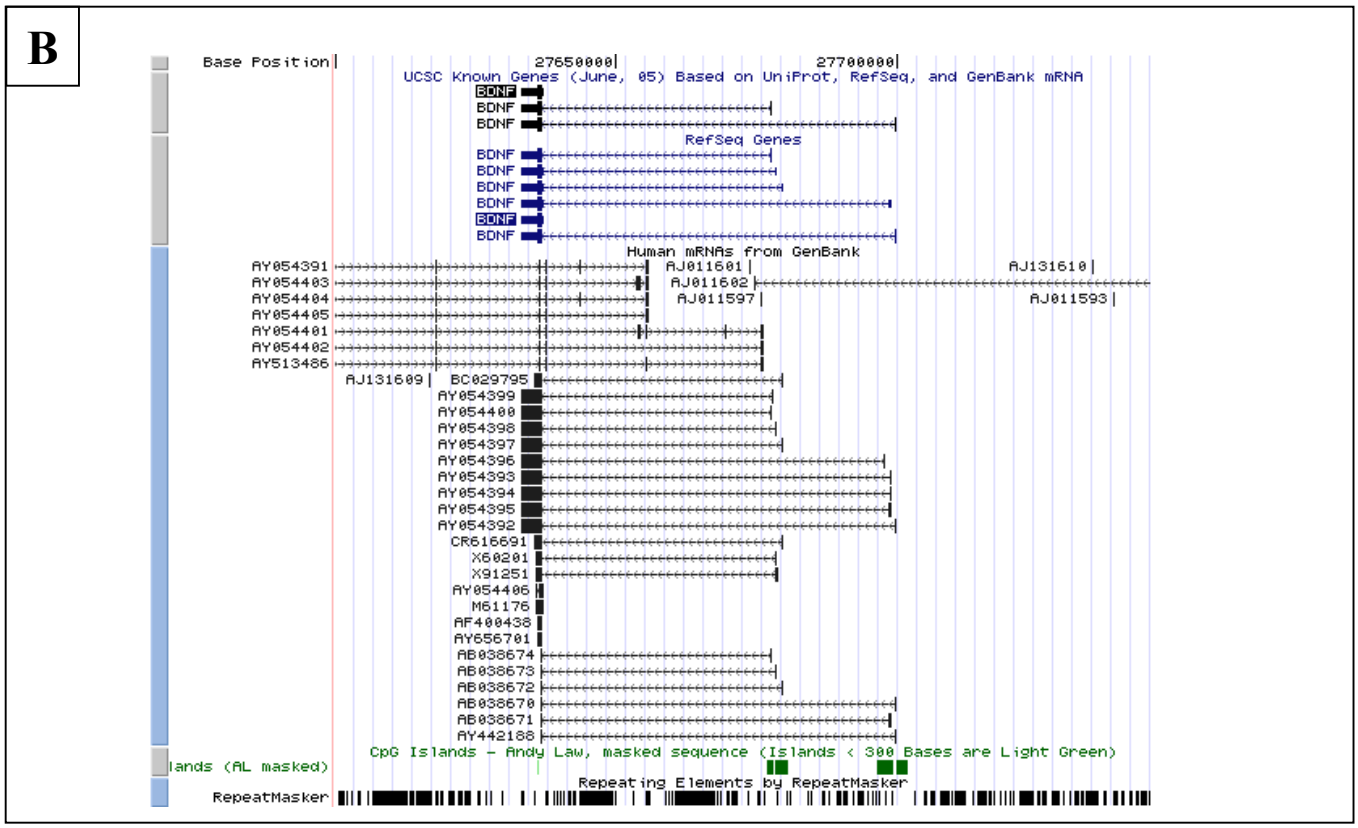
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RepeatMasker

line figure

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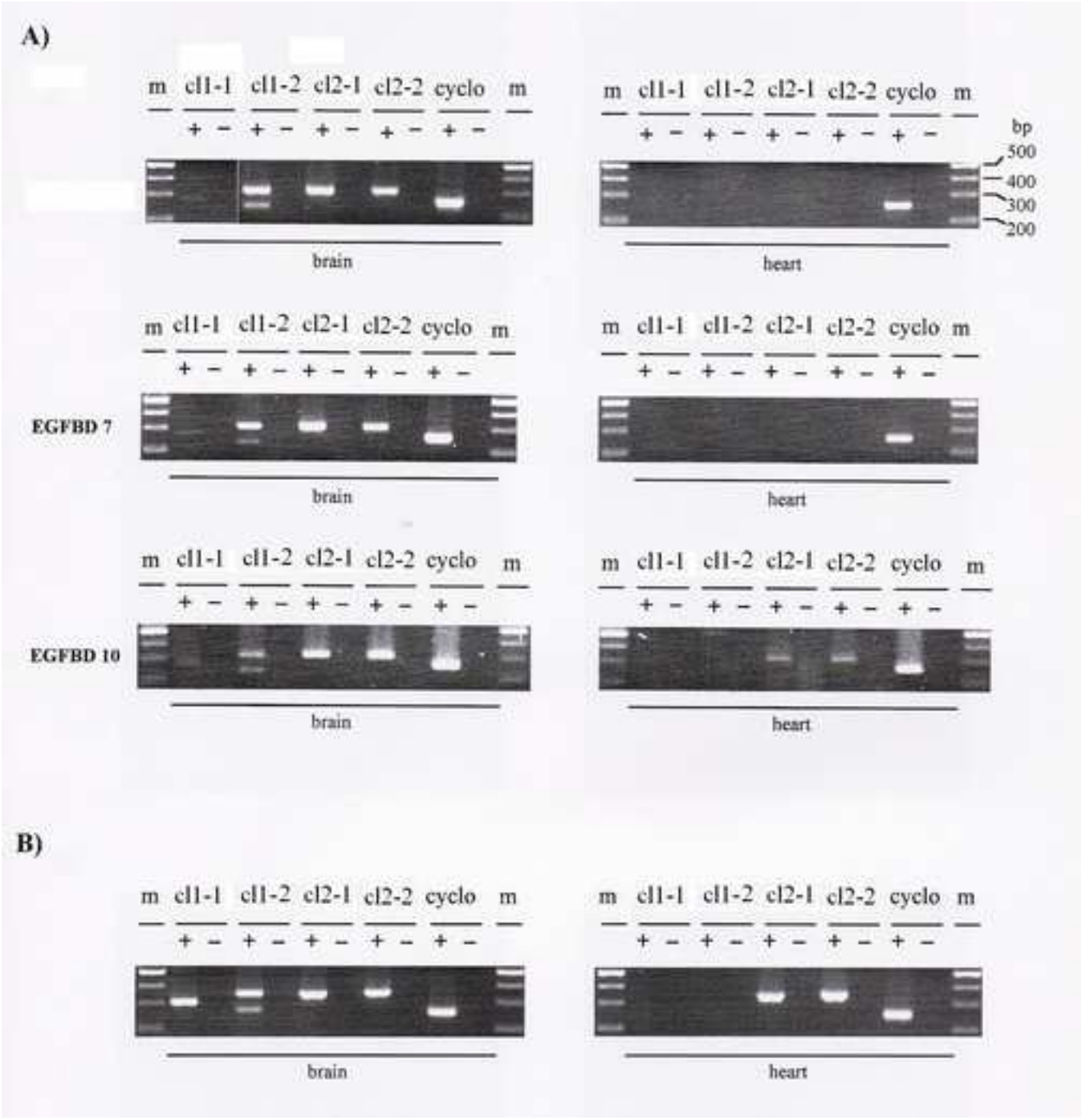

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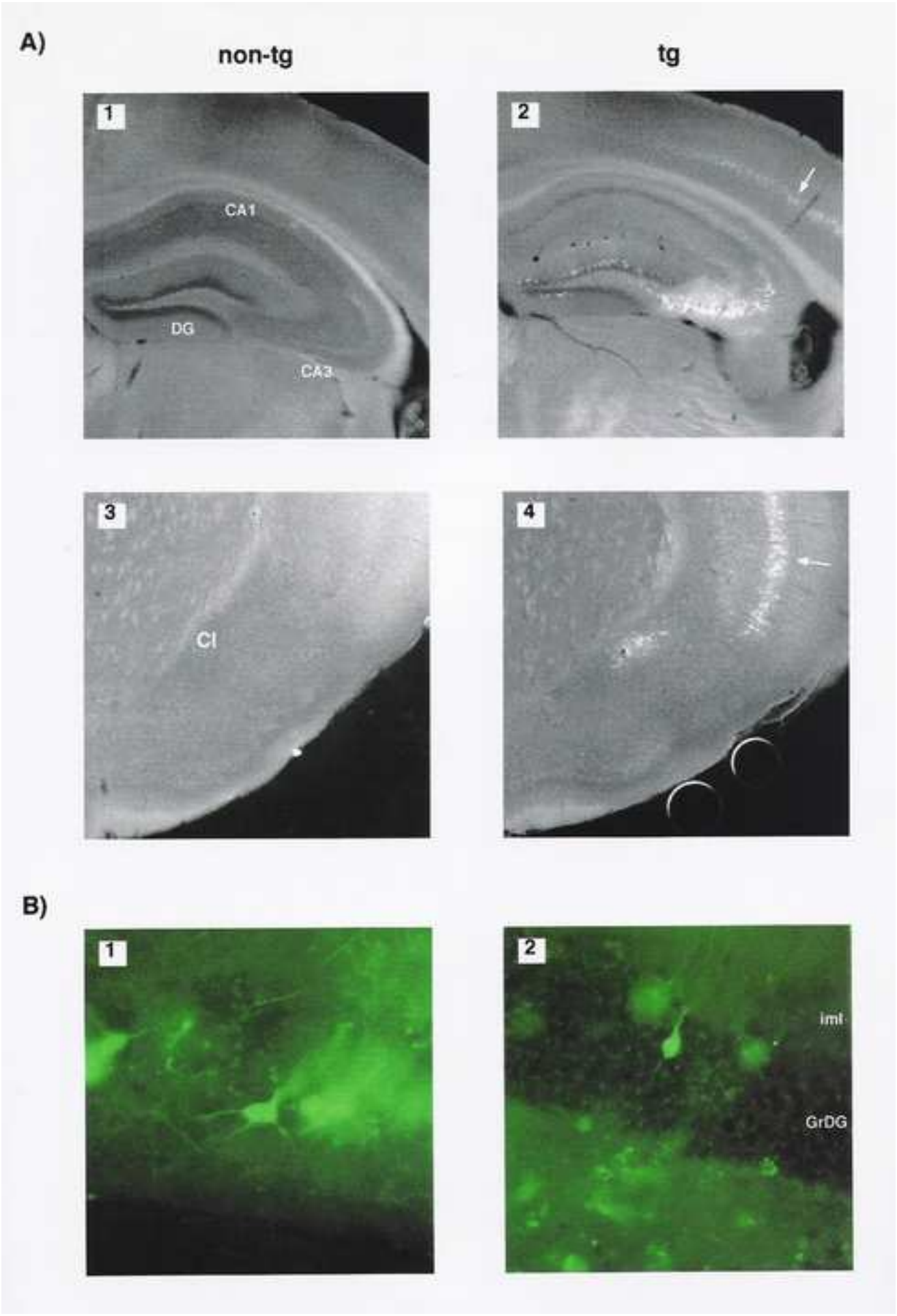

A transgenic mouse model engineered to investigate human brain-derived neurotrophic factor *in vivo*

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Abbreviations footnote : BDNF, brain-derived neurotrophic factor ; YAC, yeast artificial chromosome, E-GFP, enhanced green fluorescent protein; STS, sequence-tagged site; kb, kilobase; nt, nucleotide; PCR, polymerase chain reaction.

Keywords*:* BDNF, neurotrophic factor, YAC, transgene, expression, reporter

ABSTRACT

Brain-derived neurotrophic factor (BDNF) is an attractive component for the treatment of various neurodegenerative diseases such as Alzheimer's or Parkinson's disease. Innovative non invasive therapeutic approaches involve appropriate pharmacological induction of endogenous BDNF synthesis in brain. A transgenic mouse model has been established to study human *BDNF* gene expression and permit the screening of compounds capable of stimulating its activity. A 145-kb yeast artificial chromosome carrying the human *BDNF* gene has been engineered to produce the transgene which contains the extended *BDNF* promoter and 3' flanking regions and has integrated the enhanced green fluorescent protein (E-GFP) coding sequence in place of the *BDNF* coding exon. Five transgenic lines have been obtained through microinjection of the YAC into fertilized mouse oocytes. From the three lines expressing the transgene, one displays the specific pattern of *BDNF* expression. Faithful tissue-restricted transcription of *BDNF* 5' exons and localization of the fluorescent reporter gene product in the expected brain subregions are reported. This line constitutes an exploitable system for investigating human *BDNF* gene regulation *in vivo*.

INTRODUCTION

Brain-derived neurotrophic factor (BDNF) is a member of the neurotrophin family which groups together small basic proteic factors, structurally and functionally related to nerve growth factor (NGF) (Levi-Montalcini, 1987, Hallbook et al, 1998). These proteins display various trophic activities on specific neuronal populations during development (Snider, 1994) and in adulthood (Davies et al, 1995, Connor et al, 1998). Several features of BDNF have made this factor a very attractive candidate for therapeutic strategies against common neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease or amyotrophic lateral sclerosis (Connor et al, 1998, Yuen et al, 1996) and more recently Huntignton's disease (Ferrer et al, 2000).

In Alzheimer's disease (AD), the decrease in BDNF mRNA level observed in the hippocampus of AD patients (Phillips et al, 1991, Murray et al, 1994) may contribute to the hallmark of this pathology : progressive degeneration of the basal forebrain cholinergic neurons. Several *in vivo* experiments have shown that BDNF is required for the survival of these septal neurons (Nonomura et al, 1995, Eide et al, 1993). In addition to trophic activities, numerous recent studies have revealed BDNF functions concerning neuronal activity and plasticity (McAllister et al, 1999), reviewed in (Mattson et al, 2004). BDNF has been shown to have a central role in the formation of hippocampal long-term potentiation, in an experimental study of synaptic transmission related to learning and memory formation (Patterson et al, 1996, Ma et al, 1998). Thus the impairment of *BDNF* gene expression in AD patients' hippocampus could also directly contribute to the cognitive deterioration characteristic of AD. Furthermore, BDNF has been shown to stimulate glycolysis and glucose uptake in cortical cell cultures (Knusel et al, 1996). Thus this factor could be used as treatment for another AD feature : alteration of energy metabolism in brain cortical areas.

The critical goal a pharmacological treatment of these diseases should reach is to ensure a constant level of the neurotrophic molecule close to the sensitive neurons. Several experimental modes of administration have been described for BDNF: (i) systemic injection of a molecular reformulated BDNF (Wu et al, 1999), (ii) infection with engineered adenoviral vectors encoding this factor (Gimenez y Ribotta et al, 1997), (iii) graft of cells modified by BDNF-coding retroviral vectors (Menei et al, 1998, Martinez-

Serrano et al, 1996, Levivier et al, 1995, Girard et al, 2005). Alternatively, it may be envisioned to stimulate by appropriate drugs the production of endogenous BDNF in cells or tissues close to the site of action. This strategy has already been proposed for NGF in the case of other neurodegenerative diseases (Saporito et al, 1993, Carswell, 1993, Semkova et al, 1999). In this perspective, the generation of an animal model to study *in vivo* the regulation of the human *BDNF* gene can be considered as a valuable contribution towards finding specific compounds displaying novel therapeutic properties.

Primary descriptions of *BDNF* gene organization have been reported in rodents (Timmusk et al, 1993, Bishop et al, 1994, Hayes et al, 1997) and in human (Rosier et al, 1994), and our unpublished results). Increasing availability of genome and transcript sequences (for example on the Genome Browser web site http://genome.ucsc.edu/cgi-bin/hgGateway?) have now provided more precise pictures of BDNF gene organisation in several species. The protein is entirely encoded by a unique exon with two polyadenylation signals at the 3' untranslated end. Multiple 5' non coding exons are alternatively spliced to the coding exon. Alternative donor sites exist in the 3' region of several exons, further increasing the potential diversity of *BDNF* transcripts. In brief, two main clusters of 5' exons are detected upstream of the coding exon at distances ranging from 26 kb in rat to 41 kb in human. Isolated additional exons are also occasionnally described between the second cluster and the coding exon but they are poorly documented and have not been taken into account in this study. The two 5' clusters (about 2 to 2.5 kb each) are separated by a 15- to 17-kb intron. Each cluster contains three 5' exons (100 to 700-nt long). For sake of clarity the *BDNF* 5' exons will be numbered here according to their position in the cluster preceded by cl1 or cl2 for the first or the second cluster respectively (Figure 1). In this work, the four 5' exons mainly used in previous studies (Timmusk et al, 1995) have been selected to study BDNF gene expression. These four 5' non coding exons, initially noted I, II, III and IV in rat (Timmusk et al, 1993), correspond here to exons cl1-1, cl1-2, cl2-1 and cl2-2, described for the human *BDNF* gene in RefSeq entries NM_170731, NM_170732, NM_170733, and NM_001709 respectively. Corresponding exons are described in rat GenBank entries S76758, S76759/M67175, S76760 and S76799, as well as in mouse GenBank entries AY057908, AY057910/AY057911, AY057913 and AY057914.

Expression of the murine *BDNF* gene has been mainly detected in the central nervous system, mostly in hippocampus and claustrum (Hofer et al, 1990, Yan et al, 1997, Conner et al, 1997). In contrast

with the precise descriptions existing for *BDNF* mRNA distribution in rat brain, only limited information is available for human brain (Phillips et al, 1991, Murray et al, 1994); data concern mostly the hippocampus formation and are consistent with reports in rodents.

Transgenic models have been constructed to study the rat *BDNF* gene (Timmusk et al, 1995). Analysis of the transgenic lines has confirmed the differential use of the four promoter regions of the murine gene, depending on tissues or on various exogenous stimulations. However, the portions of the *BDNF* gene introduced in the transgenic mice appeared not to include all the regulatory elements sufficient to exactly reflect all aspects of the spatio-temporal regulation of *BDNF* gene expression. Such a difficulty observed in a transgenic model for human apolipoprotein B was circumvented by using large (145 and 207 kb) bacterial artificial chromosomes as transgenes (Nielsen et al., 1997). Yeast artificial chromosomes (YAC) can also be used as transgenes. YACs were initially designed for positional cloning (Burke et al, 1987). These vectors allow the construction of transgenes carrying very large 5' and 3' flanking regions, as well as introns, which are sometimes required to faithfully reproduce native gene expression. Accuracy of human gene expression in a mouse transgenic context has been reported in various examples (Lamb et al, 1995, Huxley, 1998, Stocksley et al, 2005). Complementation of murine genetic defects through insertion of a YAC or a BAC containing the native human homologous gene (Hodgson et al, 1996, Schedl et al, 1996, Manson et al, 1997, Sarsero et al, 2004) also indicates appropriate regulation of human genes in such transgenic mouse models.

In order to contribute to the definition of new therapeutical strategies based on the regulation of human *BDNF* gene expression, we have decided to study its expression using a YAC-transgenic model. Our approach consisted in generating transgenic mice with a 145-kb YAC including the complete human *BDNF* gene, surrounded by large genomic sequences likely to contain most regulatory signals, and in which the coding sequence of the gene was replaced by the *E-GFP* (enhanced green fluorescent protein) reporter gene (Cormack et al, 1996). Several transgenic mouse models have documented the interest of using such a reporter gene which can be readily detected by fluorescent microscopy (Ikawa et al, 1995, Zhuo et al, 1997, Godwin et al, 1998, van den Pol et al, 1998).

Five transgenic mice harboring the engineered 145-kb YAC were obtained by pronuclear microinjection in mouse oocytes and lines were established by breeding. The expression pattern of the

different transgenic transcripts was then analyzed in mouse heart and brain by RT-PCR and *in situ* detection of E-GFP fluorescence was performed on brain sections. These experiments reveal that one of the transgenic line displays a BDNF-specific pattern of transgene expression in the brain. This transgenic line constitutes the expected animal model for exploring potential pharmacological modulators of human *BDNF* gene expression.

MATERIALS AND METHODS

Materials

The 145-kb YAC containing the human *BDNF* gene used to create the transgene was obtained by fragmentation of the 810-kb mega YAC 886H12 (CEPH-Fondation Jean Dausset, Paris) as described previously (Guillemot et al, 1999). Oligonucleotides (Table 1 ; (Rosier et al, 1994, Guillemot et al, 1999)) were purchased from Life Technologies or Genosys. Mice were provided by IFFA-CREDO. Restriction endonucleases were purchased from New England Biolabs.

Retrofitting of the YAC containing the human *BDNF* gene

The reporter gene encoding the Enhanced Green Fluorescent Protein (E-GFP) (Cormack et al, 1996) was inserted into the 145-kb fragmented YAC using the two-step modification method called " pop-in, pop-out ", as previously described (Duff et al, 1994). The pEGBDPOP12 plasmid was designed for this purpose. It was created by cloning the reporter gene sequence, surrounded by two portions of the human *BDNF* gene coding exon, into the *HindIII* cloning site of the pRS406 vector (Stratagene). A first version was constructed with the GFP wild-type gene cloned into the pCX-GFP plasmid, a kind gift from M. Okabe (Ikawa et al, 1995), using fusion PCR (Uchida, 1992). On one hand, an amplified segment flanking the 5' end of the human *BDNF* coding sequence (position 291 to 668 in Genbank M61181 sequence) was fused to a 717-bp GFP amplification product from the pCX-GFP plasmid; on the other hand the same GFP amplification product was fused to an amplified segment overlapping the 3' end of the human *BDNF* coding sequence (position 1207 to 1605). Chimeric fusion primers were designed so as to contain a *SphI* site at each junction between the GFP and the BDNF sequences. After sequence checking, the two fusion products were cloned separately into PCR2.1 (Invitrogen) and combined through a common and unique *NdeI* site in the GFP sequence. The insert was then transfered into the pRS406 vector, yielding the pRS406-BDGFP plasmid. The GFP wild-type sequence was excised by *SphI* digestion and replaced by the E-GFP sequence isolated from the pEGFP-N1 vector (Clontech, Genbank accession number : U55762). The 783-bp *HindIII / NotI* fragment containing the E-GFP coding sequence was ligated into the *SphI* site of the pRS406-BDGFP plasmid with appropriate adaptors, yielding the pEGBDPOP12 plasmid. For the "pop-

in, pop-out" procedure, this plasmid was linearized by digestion with *BglII* (position 418 in Genbank M61181 sequence) and purified prior to transformation into the YAC-containing yeast strain using a lithium acetate transformation procedure (Alkali Cation Transformation kit, BIO 101) according to the manufacturer's recommendations. Proper integration and excision of the URA3 vector sequences were selected on appropriate media and checked on several YAC clones by PCR as described elsewere (Duff et al, 1994). The expected modification was assessed by Southern blot analysis of *NotI*- and *BssHII*-digested YAC DNA by pulsed-field gel electrophoresis (Guillemot et al, 1999).

Generation of transgenic mice

The 145-kb transgene was isolated by preparative pulsed-field gel electrophoresis and prepared for microinjection as described (Huxley, 1998). After concentration and dialysis, a 2 ng/µl YAC DNA solution was obtained. Transgenic mice were generated by pronuclear microinjection as described (Hogan et al, 1994). Eggs were derived from (C57BL/6J x DBA2) females mated to identical hybrid males. Founder mice were identified by PCR analysis with DNA prepared from tail biopsies, using the EGFPS and the EGFPAS primers (Table 1).

Copy number estimation

Southern blot was performed using 10 µg of mouse genomic DNA extracted from tails and digested to completion with *BanII* (Biolabs). A 886-bp *BanII* fragment containing the E-GFP sequences was excised from the pEGBDPOP12 plasmid and used as template (50 ng) to synthesize an [α-^{32}P]dCTP-labelled probe with the Random Priming Kit system (Life Technologies). Blots were hybridized for 1 h at 68°c in HybridExpress buffer (Clontech), washed following the manufacturer's recommendations and exposed on phosphor screens (Molecular Dynamics). The signals were imaged and quantitated with a Phosphorimager (Molecular Dynamics).

RNA analysis

Up to 250 mg of tissues (brain or heart) were snap frozen in liquid nitrogen for storage at -80°C or in the RNAlater solution (Ambion) for storage at -20 °C. Poly(A) RNA was extracted using the MPG Direct mRNA purification kit (Quantum/Bioprobe) according to the manufacturer's protocol. A quantity of 250 to 500 ng of poly(A) RNA was mixed with the same amount of oligod$T_{(12\text{-}18)}$ primer (Life Technologies) and

used for cDNA synthesis using the Superscript II reverse transcriptase system (Life Technologies) as described previously (Guillemot et al, 1999). For the negative control, the same amount of RNA was submitted to similar incubation conditions except for the addition of reverse transcriptase. The cDNA corresponding to 12.5 ng of poly(A) RNA was used as template for PCR amplification in a 35 µl reaction volume. Amplification reactions were performed with specific primer sets (see Table 1) in 1X PCR buffer (Platinum, Life Technologies) supplemented with 1 mM $MgCl_2$, 0.2 mM dNTPs, 1 µM of each primer and 0.25 U of the antibody-complexed Platinum Taq DNA polymerase (Life Technologies). Cycling was as follows : 2 min at 94 °C, 39 cycles (27 cycles for cyclophilin primers) composed of 30 sec at 94°C, 30 sec at 59 °C and 30 sec at 72°C, and 8 min at 72°C.

E-GFP imaging in vibratome sections

Hemizygous transgenic or control adult mice were perfused with 4% paraformaldehyde in 0.1 M PBS (pH 7.4). Brains were removed and post-fixed in the same fixative for an additional 48 h at 4°C, rinsed twice in 0.1 M PBS, embedded in 4% low melting point agarose and cut on a Leica vibratome at a thickness of 70 µm in the coronal plane. Sections were mounted in Fluoromount (Cliniscience) and E-GFP fluorescence was visualized with a fluorescence DRMB microscope (Leica) or with a fluorescence stereomicroscope MZ FLIII (Leica) combined with a cooled digital camera ORCA-100 (Hamamatsu Photonics).

PCR reactions

Mouse tail DNA lysate was prepared as follows : a piece of tail was incubated in 500 µl of lysis buffer (50 mM Tris-HCl pH 8; 100 mM NaCl; 0.5% Tween 20) supplemented with 600 µg of proteinase K (Boehringer), at 55°C overnight. The lysate was then centrifuged and 1 µl of the supernatant was used as template in a 25 µl PCR reaction volume using the Ready-To-Go kit (Pharmacia Biotech) in the presence of the EGFPS and EGFPAS primers (1 µM of each). Cycling conditions were as follow : 1 min at 94°C, 29 cycles composed of 1 min at 94°C, 1 min at 60°C, 1 min at 72°C and 10 min at 72°C. Except for the annealing temperature (Table 1), the same cycling conditions were applied for amplification with the HIS3 primer set which corresponds to a portion of the YAC right arm. In this case, the reaction was performed with the BioTaq DNA polymerase (0.5 U, Eurobio) in the corresponding buffer supplemented with 1.5 mM $MgCl_2$, 1 µM of each primer and 0.2 mM dNTPS. For the other YAC-specific STSs (886H12L, A2C4,

B2F11, C2C4 and C2D9) which are described in Guillemot et al. (1999), amplifications were performed in the same reaction conditions but with another cycling program: 2 min at 94°C, 30 cycles composed of 30 sec at 94°C, 30 sec at 59°C, 30 sec at 72°C and 8 min at 72°C.

RESULTS

Construction of the YAC transgene

A 145-kb YAC derived from the CEPH 886H12 YAC (42) was modified to produce the transgene. This YAC (Figure 1A) carries the human *BDNF* gene spanning 67 kb (distance on the genome from the beginning of the most upstream 5' exon to the last nucleotide of the longest version of the coding exon), flanked by about 35 kb downstream of the coding exon and 45 kb upstream of 5' exons cluster 1. Approximate mapping of the *BDNF* gene on the YAC results from both previous restriction map analysis (Rosier et al, 1994) and localisation of the *NotI* and *BssHII* sites between exon III and IV and close upstream of the coding exon respectively (Figure 1A). The genomic coordinates of the YAC insert on the human genome sequence (NCBI Build 35) can thus be approximated to chr11:27 600 000-27 745 000. A picture of the genomic context of the *BDNF* gene in this region can be obtained from the Genome Browser (http://genome-test.cse.ucsc.edu/cgi-bin/hgTracks) and is represented in Figure 1B. Several mRNAs corresponding to a poorly characterized gene transcribed from the opposite DNA strand overlap the coding region and last intron of the *BDNF* gene. No promoter region other than the one corresponding to the *BDNF* gene seems to be present in the YAC, as only two groups of CpG islands are nicely co-localized with the two clusters of 5' exons of the *BDNF* gene. In addition to the *BDNF* gene, we have previously identified and mapped on this YAC the four sequence-tagged sites (STSs) C2D9, B2F11, C2C4 and A2C4, corresponding to human 3' terminal sequences (AJ011602, AJ011597, AJ011601, and AJ011593 respectively) isolated by an exon trapping procedure (Guillemot et al, 1999).

YAC modification consisted in the partial replacement of the coding exon of the *BDNF* gene by the reporter gene encoding the enhanced green fluorescent protein (E-GFP). Three bases downstream the translation initiator codon of the *BDNF* gene, a 540-bp exonic portion was replaced by a 783-bp plasmidic sequence containing the reporter gene (Figure 1A). The substitution was performed using a method based

on homologous recombination called "pop-in, pop-out" (Duff et al, 1994), which required construction of a plasmid bearing the expected modification (see Materials and Methods). Expression of the reporter gene product from this construction was checked by subcloning the substituted *BDNF* coding exon into the pCDNA3 expression vector (Invitrogen) and observing fluorescence upon transfection into COS cells (not shown). The "pop-in, pop-out" procedure yielded a YAC clone harboring the expected features, that was characterized by PCR and Southern blot analysis. The E-GFP sequence was shown to be present in the 80-kb *NotI* fragment containing the coding exon of the *BDNF* gene and amplification experiments using primers specific for *E-GFP* and *BDNF* sequences confirmed that the replacement had been correctly done (data not shown). This resulting recombinant YAC, named 886EGFBD, was used as transgene.

Generation of transgenic mice

DNA from the recombinant 886EGFBD YAC was gel purified and microinjected into fertilized oocytes that were transplanted into foster mothers. From 320 injected eggs, 18 live born animals were obtained. Tail DNA from this progeny was screened using primers specific for the *E-GFP* reporter gene and five positive founder mice were identified. Transgenic lines were derived from the breeding of founders with non-transgenic C57BL/6J mice. To assess transgene integrity, mouse genomic DNA was also tested with six other PCR markers that span the entire length of the YAC (Figure 1A). All of them were detected in the genomic DNA of all founder animals and were transmitted to the offspring. Interestingly, in one of these lines (called EGFBD11) transgene transmission was restricted to males, suggesting integration in the murine Y chromosome.

As a first screening for accurate transgene expression, detection of transgenic transcripts containing *BDNF* exon cl1-2 was performed by RT-PCR using poly(A) RNA prepared from brain of mice from the five transgenic lines. *BDNF* exon II is known to be expressed specifically in brain. The expected transcript was detected only in three transgenic lines (named EGFBD3, EGFBD7 and EGFBD10) which were further analyzed. Southern blotting was performed to determine the number of transgene copies integrated in the genome of the transgenic mice (data not shown). The EGFBD3 mice integrated two or three copies of the YAC whereas a higher copy number was detected for the EGFBD7 and EGFBD10 lines (about 7 and 6 copies respectively).

Transcript analysis

After testing the presence of the transgenic transcripts containing human *BDNF* exon cl1-2 in brain, RT-PCR assays were performed to detect the transgenic transcripts containing the three other 5' exons (cl1-1, cl2-1 and cl2-2). For all lines analyzed, a signal can be detected for each type of transcript in brain (Figure 2A, left panels). This expression pattern reproduces the pattern of endogenous *BDNF* gene in this organ (Figure 2B, left panel). Interestingly, in addition to the 315-bp band expected for transgenic cl1-2 transcript, a smaller product was also produced upon amplification in the presence of exon-cl1-2 sense primer (Figure 2A, lanes cl1-2 +). This product was cloned and its sequence revealed that is corresponds to an alternative splicing form of exon-cl1-2 transcript in which 83 nt are skipped at the 3' end of exon cl1-2. Indeed, the existence of three alternatively spliced exon-cl1-2 transcripts has recently been observed (Liu et al, 2005) upon sequencing of various BDNF isoform mRNAs and confirms our observation (isoforms 2A : AY054393, 2B : AY054394, and 2C : AY054395, this last one being truncated by 83 nt to produce isoform 2B). A similar amplification product is visible upon amplification of mouse endogenous exon-cl1-2 transcript (Figure 2B, lane cl1-2 +). Sequence comparison between the murine and human transgenic truncated products revealed that they correspond in both species to the usage of an alternative donor splice site located at the same position in exon cl1-2 (unpublished results). Here again, sequences available for *BDNF* exon-cl1-2 transcripts in mouse (Liu et al, 2006) confirm the existence of three isoforms very similar to the three isoforms observed in human.

In order to study the expression of the various transgenic transcripts in a non-neuronal tissue, RT-PCR assays were then implemented with poly(A) RNA prepared from heart (Figure 2, right panels). It has been established previously that transcripts containing exons cl1-1 or cl1-2 of the rat *BDNF* gene are not expressed in heart whereas exon-cl2-1 and –cl2-2 promoters are active in this organ (Timmusk et al, 1995). Our results are in good agreement with this observation since no signal was detected in non-transgenic mouse heart for murine exon-cl1-1 and –cl1-2 transcripts, whereas murine exon-cl2-1 and -cl2-2 transcripts were shown to be expressed in heart (Figure 2B, lanes cl1-1 to cl2-2). Of the three transgenic lines analyzed, only line EGFBD10 displayed expression of transgenic exon-cl2-1 and –cl2-2 transcripts in heart (Figure 2A, lanes cl2-1 and cl2-2). Expression of murine exon-cl2-1 and –cl2-2 transcripts was

found positive in all analyzed heart samples from EGFBD3, EGFBD7 and EGFBDD10 lines (data not shown). However the same heart RNA samples failed to give any signal for the corresponding transgenic transcripts in EGFBD3 and EGFBD7 lines.

In conclusion, a transgenic expression pattern concordant with that of the endogenous *BDNF* gene in mouse brain and heart was detected only in line EGFBD10.

E-GFP detection in the brain

In order to analyse transgene expression *in situ*, vibratome sections were performed on brain from transgenic and non transgenic adult mice and the slices were illuminated with appropriate UV light to visualize E-GFP fluorescence. Detection analysis was focused on coronal sections at the level of the hippocampal formation and the claustrum, two cerebral areas where the highest levels of reporter gene expression were expected. In brain sections from EGFBD3 and EGFBD7 mice, no specific E-GFP signal could be distinguished from the non-transgenic tissue autofluorescence (data not shown). By contrast, in the EGFBD10 line, cells showing strong E-GFP fluorescence were detected in the hippocampus and claustrum (Figure 3A, panels 2 and 4).

In the hippocampus (Figure 3A, panel 2), cells expressing E-GFP at high level were localized in the dentate gyrus and in the CA3 region of Amon's horn. Transgene expression was not detected in CA1. In Amon's horn, the E-GFP labeled cells were found in the pyramidal cell layer. In the dentate gyrus, transgene expression was detected in cells of the stratum granulosum, mainly localized close to the separation with the inner molecular layer (Figure 3B, panel 2). The neuronal nature of these labeled hippocampal cells was assessed by immunohistochemical detection of the neuron-specific marker MAP2 (data not shown). Intracellular distribution of E-GFP appeared rather homogenous and allowed to reveal the cellular morphology (cell bodies and neurites) of these labeled neurons (Figure 3B, panels 1 and 2). In the claustrum, high levels of E-GFP were also detected (Figure 3A panel 4) in clearly stained cell bodies.

In addition to the hippocampus and claustrum, E-GFP expression was also observed in the neocortex (Figure 3A, panels 2 and 4, arrows). Localization of the labeled neurons seemed to be restricted to an intermediate layer in the parietal cortex. Transgene expression was further analyzed on several

sections throughout the brain and cerebellum of mice from the EGFBD10 line but these observations failed to reveal any additional structure producing detectable levels of E-GFP.

Taken together these results show that in the transgenic line EGFBD10, the human *BDNF* promoter is able to drive specific expression of the *E-GFP* reporter gene and that the obtained pattern is restricted in brain to subsets of neurons from the hippocampus, claustrum and parietal part of the brain cortex.

DISCUSSION

The work reported here aimed at assembling together various features to produce an appropriate model to study potential modulators of human *BDNF* gene expression *in vivo*. (i) The model is an animal model so as to gain access to all stages and places of expression of the gene. In addition, the transgenic mouse line thus obtained constitutes an available source of various cell types containing the same recombinant construction. (ii) The *BDNF* promoter considered is of human origin to increase the chance that the results of the study can be used for therapy of human diseases. This promoter is handled in a YAC to retain a large portion of the regulatory sequences around the human *BDNF* gene. (iii) A reporter-gene coding sequence has been inserted in the *BDNF* coding exon to trace the activity of the gene. This reporter coding sequence (about 780 bp) is similar in size to the replaced sequence of the *BDNF* coding exon (540 bp). Thus this substitution does not alter the overall structure of the *BDNF* gene, and probably affects in a very limited way the structure of its mRNA. (iv) The reporter gene encodes E-GFP with fluorescent properties used in many living cells or organisms (Cubitt et al, 1995, Misteli et al, 1997), a feature which may allow straightforward development of novel detection systems and high-throughput screening procedures (Gervaix et al, 1997).

Microinjection of the 145-kb engineered YAC into fertilized mouse oocytes resulted in an efficiency of 27 % (5/18) of transgenesis among all born animals. This is very similar to the efficiencies obtained with constructions of more limited size. The genome of the five transgenic lines obtained contained intact YAC

DNA as shown by monitoring STS content, suggesting that few if any deletion occurred during transgenesis. As observed in other cases (Lamb et al, 1995), pronuclear microinjection proved to be a straightforward method to produce YAC transgenic mice. Despite the fact that the preparation and manipulation of high-molecular weight DNA prior to the microinjection are very demanding steps, this method has been used successfully for YAC DNA up to nearly 700 kb (Smith et al, 1995).

Three from the five transgenic lines obtained expressed the transgene in brain. This is less than expected when considering the large size of the transgene. Indeed, it is generally assumed, and it has been confirmed in many cases, that large DNA segments will contain the necessary signals, such as matrix attachments regions (MAR), to insulate active loops of chromatin and thus annihilate position effects in transgenic models (McKnight et al, 1992, Namciu et al, 1998). The MAR elements, ranging from 100 to 1000 bp, are estimated to be present in about 100,000 copies in mammalian genomes (Bode et al, 1996), i.e. one every 30 kb in the human genome if evenly spaced. Thus, the likelihood that one or more MAR exist in the inserted 145-kb transgene is high. Nevertheless, the functions of MARs are very diverse and some cases have already been reported where insertion of a MAR element on both sides of a transgene is not sufficient to ensure position-independent and/or faithful tissue specific expression (Barash et al, 1996, Neznanov et al, 1996). In our study, persisting positional effects may also result from some as yet undescribed dominant effects of mouse MARs on one or both sides on the transgene.

Analysis of tissue-specific expression of the transgene revealed that two from the three lines expressing the transgene in brain did not express the transgenic exon-cl2-1 and -cl2-2 transcripts in heart. This defect in the pattern of expression is likely correlated to the lack of detection of the reporter gene product *in situ* for these two lines. Indeed, although the presence of a transcript was clearly established by RT-PCR performed on total brain mRNA (figure 2A, left panels), it was impossible to detect any well-defined regions of fluorescence in the brain of these transgenic animals, in particular in the hippocampus known as the main site for *BDNF* gene expression. Transcription levels may be insufficient and/or presenting a too diffuse distribution in brain to produce enough E-GFP for detection. The use of improved detection techniques allowing to increase the ratio between specific E-GFP signals and background autofluorescence should help testing this hypothesis. Putative position effects are likely responsible for this situation and could be investigated using larger (>145 kb) transgene constructs. Indeed, the large size of

the *BDNF* gene itself (~ 67 kb) and its position in the YAC utilized here are limiting the length of available regulatory regions on each side of the gene (45 and 35 kb upstream and downstream respectively). Longer YACs extending further upstream the transgene have been produced through fragmentation of the 810-kb initial YAC (Guillemot et al, 1999) and could be used in future experiments to increase the chance to get position-independent expression and appropriate tissue distribution of the transcript.

Finally, *in situ* detection of the fluorescent reporter gene product was successful for one of the transgenic line. In this line, the restricted pattern of expression observed in the hippocampus, claustrum and cortex is very consistent with previous reports in rat brain (Yan et al, 1997, Conner et al, 1997). In primate, hippocampus is found as the major site of detection of *BDNF* mRNA (Phillips et al, 1991, Murray et al, 1994) and protein (Hayashi et al, 1997, Murer et al, 1999). Our transgenic model allows easy *in situ* visualization of the human *BDNF* gene activity and may thus become a valuable tool to further investigate the human *BDNF* gene regulation *in vivo*.

A YAC-transgenic mouse line correctly expressing E-GFP protein under the control of the human *BDNF* gene is now available. Further characterization should lead to develop a high throughput screening assay for pharmacological substances capable of modulating *BDNF* gene expression *in vivo*. Proper regulation of the gene can first be checked using various neurotransmitters analogs and hormones already known as potent stimulators of *BDNF* transcription *in vivo* such as agonists for NMDA-glutamate (da Penha Berzaghi et al, 1993), cholinergic muscarinic and serotonin $5HT_{2A/2C}$ receptors (Vaidya et al, 1997), antagonists for $GABA_A$ receptors (Metsis et al, 1993), estradiol (Singh et al, 1995) and AVP(4-8) peptide (Zhou et al, 1997). Cell culture systems can then be established from the transgenic animals, in which activity of the *BDNF* promoter could be monitored by fluorescence measurement of the reporter gene product following application of series of pharmacological substances. The identified active compounds would then undergo whole animal assays to check for *in vivo* efficiency and absence of secondary effects. The therapeutic value of such compounds could then be tested in animal models of neurodegenerative diseases prior to entering clinical trials with human patients. In conclusion, the availability of a transgenic mouse model in which faithful brain-specific expression of a human BDNF-GFP transgene is achieved

opens a large field of potential discoveries which may lead to innovative strategies for the treatment of neurodegenerative diseases.

ACKNOWLEDGEMENTS

We wish to thank Esther Toselli and Cécile Hervieux for DNA sequencing, Arlette Loeuillet, René Papion-Duchateau, Jean-Paul Moussu and Patrice Medina from SEAT for technical assistance in trangenesis and mouse manipulation, Nathalie Spassky and Bernard Zalc (INSERM U-495, Hopital de la Salpétrière, Paris) for help in E-GFP visualization, MAP2 detection and interest for this work. We are indebtful to Clare Huxley for advices and sharing protocols. This work was supported by grants from Association Française contre les Myopathies (AFM) and Ligue Nationale contre le Cancer. The authors are grateful to Association France-Alzheimer for interest and financial support provided to FG. FG also benefited from fellowships from AFM, Ligue Nationale contre le Cancer, Association pour la Recherche contre le Cancer and from Cold Spring Harbor Laboratory concerning the "YACs in Structural & Biological Genome Analysis" course.

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LEGENDS TO THE FIGURES

Figure 1 : A. Description of the YAC transgene. The exons of human *BDNF* gene (symbolized by shaded boxes) are localized on the 145-kb YAC. The two clusters of 5' exons are labeled as cl1 and cl2 respectively. Exons cl1-1, cl1-2, cl2-1, cl2-2, corresponding to rat exons I, II, III and IV and analysed in this study are identified. Rare cutter restriction sites are indicated (B : *BssHII*, N : *NotI*). The grey arrows and circle indicate respectively the arms and the centromere of the YAC vector. The relative positions of the seven STSs which were shown to be present in all transgenic lines are indicated with dark triangles below the YAC map. A magnification of the YAC area where the *BDNF* coding exon (shaded box) has been partially replaced by *EGFP* reporter gene (hatched box) is shown above the map. The numbers correspond to positions on the *BDNF* gene partial sequence from Genbank M61181entry. Translation initiation codons (ATG) of the *BDNF* and *E-GFP* sequences are indicated as well as termination codons (TAG and TAA respectively).

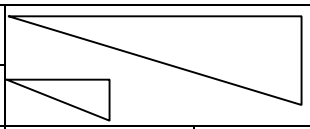
B. Genomic context. This picture was returned from the Genome Browser web server and corresponds to bases 27 600 000 to 27 745 000 from human chromosome 11, NCBI build 35, assembly April 2004.

Figure 2 : RT-PCR analysis of transgene expression in brain and heart of transgenic mice (A), and comparison with endogenous *BDNF* gene expression in non transgenic mice (B). RT-PCR experiments were performed in the presence (+) or in the absence (-) of reverse transcriptase. PolyA+ RNA was extracted from brain and heart of transgenic (EGFBD3, EGFBD7 and EGFBD10 lines, panel A) and non transgenic (panel B) mice. Sense primers specific for each 5' exon of human and mouse *BDNF* genes were used together with an antisense primer specific of either *E-GFP* (EGFPAS2, panel A) or mouse *BDNF* coding exon (mBDcodAS, panel B). The sense primers were the same for human and mouse exons cl1-1, cl1-2 and cl2-1 (hBDNFIS, hBDNFIIS and hBNDFIIIBS respectively) but differed for exon cl2-2 because of too many mismatches between the human and mouse sequences (hBDNFIVS and rBDNFIVS for human and mouse exon cl2-2 respectively). See Table 1 for primer sequences and product sizes. Amplification with specific primers for the cyclophilin gene transcript (mCYCLOS and mCYCLOAS,

Table 1) was performed as a positive control (cyclo) to check the amount of cDNA in the samples. m is the 100 pb ladder DNA size markers from Fermentas.

Figure 3 : E-GFP *in situ* detection in brain of EGFBD10 mice. Coronal sections through the hippocampus and the claustrum of non transgenic (non-tg : A, panels 1 and 3) and transgenic (tg : A, panels 2 and 4 and B, panels 1 and 2) mice were analyzed by fluorescence microscopy with filters designed for E-GFP detection (GFP3 filter set, Leica). Low-magnifications images (A) were obtained using a cooled digital camera fitted to a fluorescence stereomicroscope. Autofluorescence in the brain sections was similar in transgenic and non transgenic mice. Integration was used during image acquisition to optimize the detection of the E-GFP signal. High-magnification photomicrographs (B) were obtained by direct visualization under a fluorescence microscope. In the hippocampal structure (A, panel 2), a high E-GFP expression is clearly detected in the CA3 region of Amon's horn and in the dentate gyrus (DG). At higher magnification, the E-GFP-labeled neurons are localized in the pyramidal cell layer of CA3 (B, panel 1). In the dentate gyrus (B, panel 2), the E-GFP is expressed in a population of neurons from the granular cell layer (GrDG), close to the inner molecular layer (iml). The claustrum (Cl) also contains cells expressing a high level of E-GFP (A, panel 4). In addition to these internal structures, E-GFP fluorescence was also detected in an intermediate layer of the parietal cortex (A panels 2 and 4, arrows).

Table 1. List of oligonucleotides used in this study.

primer name	sequence 5'-3'	product size (bp)	
transgene analysis			
HIS3S*	GAT GAC AGA GCA GAA AGC CC	524	
HIS3AS*	GGA GGG TAA TTC TGC TAG CC		
EGFPS	TCG AAT TCT GCA GTC GAC GG	454	
EGFPAS	GTC CTC CTT GAA GTC GAT GC		
expression analysis			
EGFPAS2	ACT TGT GGC CGT TTA CGT CG		
mBDcodAS	CCA GTG ATG TCG TCG TCA GAC		
hBDNFIS	GGA ACT TCT CAC ATG ATG ACT TCA AAC	311	276
hBDNFIIS	TGA GCT CGC TGA AGT TGG CTT	350	315
hBDNFIIIBS	TAC CGG GCA CCA AAG ACT CG	347	317
rBDNFIVS	GCT TTG ATG AGA CCG GGT TC	356	-
hBDNFIVS	GCT TTA ATG AGA CAC CCA CC	-	313
mCYCLOS †	CGA GCT CTG AGC ACT GGA GAG AAA	260	
mCYCLOAS †	TCC AGC CAT TCA GTC TTG GCA GTG C		

S : sense, AS : antisense; * and † : the annealing temperature was 55°C and 60°C respectively, in contrast to 59°C for all other primers.